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Sex Influences the Effect of a Lifelong Increase in Serotonin Transporter Function on Cerebral Metabolism

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Polymorphic variation in the human serotonin transporter (SERT; 5-HTT) gene resulting in a lifelong increase in SERT expression is associated with reduced anxiety and a reduced risk of affective disorder. Evidence also suggests that sex influences the effect of this polymorphism on affective functioning. Here we use novel transgenic mice overexpressing human SERT (*h*SERT OVR) to investigate the possible influence of sex on the alterations in SERT protein expression and cerebral function that occur in response to increased SERT gene transcription. SERT binding levels were significantly increased in the brain of *h*SERT OVR mice in a region-dependent manner. The increased SERT binding in *h*SERT OVR mice was more pronounced in female than in male mice. Cerebral metabolism, as reflected by a quantitative index of local cerebral glucose utilization (iLCMRglu), was significantly decreased in many brain regions in *h*SERT OVR female as compared with wild-type female mice, whereas there was no evidence for a significant effect in any region in males. The ability of *h*SERT overexpression to modify cerebral metabolism was significantly greater in females than in males. This effect was particularly pronounced in the medial striatum, globus pallidus, somatosensory cortex, mamillary body, and ventrolateral thalamus. Overall, these findings demonstrate that the influence of a lifelong increase in SERT gene transcription on cerebral function is greater in females than in males and may relate, in part, to the influence of sex on genetically driven increases in SERT protein expression. © 2009 Wiley-Liss, Inc.

Key words: mice; anxiety; 2-deoxyglucose; transgenic

5-Hydroxytryptamine (5-HT; serotonin) is implicated in the regulation of emotion and in the etiology of affective disorders. The serotonin transporter (SERT; 5-HTT) regulates 5-HT availability through reuptake of the neurotransmitter into the presynaptic terminal. A number of polymorphisms in the human SERT gene (SLC6A4; *h*SERT) have been found to regulate SERT protein expression and function. One such polymorphism, a 44-bp insertion/deletion (the ''long'' [l] and ''short'' [s] allele, respectively) in the promoter (5-HTTLPR) region of the gene results in differential levels of SERT expression. Cells expressing two copies of the ''l'' allele show increased SERT mRNA levels and a 2-fold increase in 5-HT uptake when compared with those with one or two copies of the ''s'' allele (Lesch et al., 1996). Human individuals homozygous for the ''l'' allele also display increased SERT mRNA, binding sites, and 5-HT reuptake when compared to ''s'' allele homozygotes (Hanna et al., 1998; Little et al., 1998; Greenberg et al., 1999; Heinz et al., 2000). Interestingly, individuals homozygous for the ''l'' allele also exhibit decreased anxiety (Lesch et al., 1996; Greenberg et al., 2000; Schinka et al., 2004) and are less likely to experience depression (Furlong et al., 1998; Mann et al., 2000; Lotrich and Pollock, 2004; Caspi et al., 2003) than those homozygous for the ''s'' allele.

Evidence suggests that sex modulates the influence of this polymorphism on affective functioning, although there is inconsistency between studies. In females, the ''s'' allele has been reported to increase the risk of depressive symptomology (Brummett et al., 2008; Grabe et al., 2005; Sjoberg et al., 2006), while no such effect was found in males. In contrast, several studies report the opposite (Du et al., 2000; Mizuno et al., 2006) or no (Ball et al., 1997; Gelernter et al., 1998; Greenberg et al., 2000) sex effect. Data from transgenic animal studies are more consistent, where genetically determined

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alterations in SERT expression produce a greater effect in females. For example, in SERT knockout mice (SERT KO), the resulting alterations in anxiety-like behavior (Holmes et al., 2003) and serotonin system functioning (Li et al., 2000; Kim et al., 2005) are more pronounced in females. However, it could be argued that the sex effects found in SERT KO mice, where SERT function is completely abolished, may be more pronounced than those in response to more subtle, naturally occurring variations in SERT function, such as those found in humans.

Recently, a novel mouse has been genetically engineered to overexpress *h*SERT (*h*SERT OVR), resulting in an approximate 2-fold increase in SERT protein expression (Loder et al., 2000; Jennings et al., 2006). This animal model allows for the investigation of the possible modulatory influence of sex on the phenotypic and endophenotypic outcomes of a subtle alteration in SERT protein expression. In the present study, we investigate whether sex influences the increase in SERT protein expression found in the brain of *h*SERT OVR mice. Furthermore, we examined constitutive cerebral metabolism in *h*SERT OVR mice, as reflected by an quantitative index of local cerebral glucose utilization (iLCMRglu, Dawson et al., 2008), and we also determine whether the modification of cerebral metabolism by a lifelong increase in SERT expression is influenced by sex.

MATERIALS AND METHODS

Animals

All experiments were subject to local ethical review and were conducted in accordance with the UK Animals (Scientific Procedures) Act (1986) and European Communities Directive 86/609. A colony of *h*SERT OVR mice was generated as previously described (Loder et al., 2000; Jennings et al., 2003), and all experiments included both male and female heterozygous transgenic (*h*SERT OVR) mice and their wild-type littermates (CBAxC57BL/6J background, aged 2–4 months). Animals were group housed (5–6 animals per cage) under strict environmental conditions with a 12-hr light–dark cycle (lights on 0700) and a room temperature of 21°C ± 1°C. Food and water were available ad libitum until the night before the experiment, at which point access to food was restricted overnight (12 hr before experimental manipulation) for animals involved in the 2-deoxyglucose imaging study to obviate any possible hyperglycemia during the experimental procedure. At approximately 3 weeks before the start of the experiments, and again at 1 week, vaginal smears were taken and assessed by trained animal technicians, and the mice were found to be cycling synchronously with an estrus cycle of 4 days. Thus, tissue collection for the paroxetine binding study and in the determination of iLCMRglu was spread across 4 consecutive days in order to control for the possible influence of the estrus cycle in females on these parameters. Within each group ($n = 6$), no one day of the estrus cycle was represented in any more than two mice.

Measurement of SERT Binding Sites

Brains from *h*SERT OVR mice (male $n = 6$, female $n = 6$) and their wild-type littermates (male $n = 6$, female $n = 6$) were rapidly dissected out and frozen in isopentane at −40°C and stored at −80°C before sectioning. Coronal sections (20 μm) were cut in a cryostat (−20°C) and thaw-mounted onto poly-L-lysine-coated slides (VWR, UK). Two consecutive sections were mounted onto separate slides for the duplicate determination of total binding, and a third adjacent section was mounted onto a separate slide for the assessment of nonspecific binding. Sections were allowed to dry at room temperature before being stored at −80°C until the day of the experiment.

On the day of the experiment, tissue sections were removed from the freezer and allowed to equilibrate to room temperature for 30 min. Slides were preincubated in 50 mM Tris-HCl buffer (120 mM NaCl, 5 mM KCl, pH 7.4, 25°C) for 2 × 5 min. For the determination of total binding, sections were incubated in the same buffer described above but containing 0.25 nM [^{3}H]paroxetine (Amersham Bioscience, UK, specific activity 19.1 Ci/mM) for 2 hr (25°C). Nonspecific binding was determined by exposing sections to the same incubation conditions in the presence of 4 μM citalopram (Sigma-Aldrich, UK). Sections were then washed in 50 mM Tris-HCl buffer (4 × 30 min) at 4°C before being dipped in ice-cold distilled water and rapidly dried under a stream of cold air.

Slides were apposed to X-ray film (Kodak, SB-5) for 6 weeks at −40°C along with a set of precalibrated tritium standards ([^{3}H] microscales, 0.07–33.6 nCi/mg tissue equivalents, Amersham International, UK) and films were developed in accordance with the manufacturer's instructions. Autoradiographic images on x-ray film were analyzed by a computer-based image analysis system (MCID, Imaging Research Inc.). The tissue-equivalent [^{3}H] concentration of each brain region of interest (RoI) was calculated from the mean optical density of six bilateral measurements, and the optical density–[^{3}H] calibration curve was generated from the coexposed standards. The concentration of [^{3}H]paroxetine bound to SERT in each brain region was then calculated by subtracting the value of nonspecific binding from the mean total binding (determined from duplicate measurement) with reference to the specific activity (19.1 Ci/mM) of the radioligand. Throughout these studies, there was no evidence of a significant difference in the level of nonspecific binding between either sex or genotype in any RoI. This supports the suggestion that differences found in [^{3}H]paroxetine binding between both sex and genotype are due to alterations in the specific binding of this radioligand to SERT.

Measurement of iLCMRglu

Constitutive iLCMRglu was determined in a total of 23 wild-type (male $n = 11$, female $n = 12$) and 23 *h*SERT OVR (male $n = 11$, female $n = 12$) conscious mice by the adapted and validated 2-deoxyglucose autoradiographic imaging technique previously developed in this laboratory (Dawson et al., 2008). Mice were injected intraperitoneally with 5 μCi of [^{14}C]-2-deoxyglucose in 0.4 ml of saline at a steady rate

$$\text{iLCMRglu} = \left(\frac{C_i^*(T)}{\left(C_p^*(t)/C_p(t)\right)} \right) \left(\frac{\left(C_p^*(t)/C_p(t)\right)_{\text{individual}}}{\text{mean}\left(C_p^*(t)/C_p(t)\right)_{\text{group}}} \right) \left(\frac{\text{mean}\left(C_p^*(t)/C_p(t)\right)_{\text{group}}}{\text{mean}\left((C_p^*(t)/C_p(t)\right)_{\text{control group}}} \right)$$

Fig. 1. Operational equation for the determination of iLCMRglu. $C_i^*(T)$ represents the total [^{14}C] present in a single brain region. $C_p^*(t)$ indicates the concentration of [^{14}C]-2-DG, and $C_p(t)$ indicates the concentration of glucose present in the terminal blood sample. $(C_p^*(t)/C_p(t))_{\text{individual}}$ represents the terminal plasma [^{14}C]/[glucose] ratio for an individual animal, $\text{mean}(C_p^*(t)/Cp(t))_{\text{group}}$ represents the mean plasma ratio of the appropriate experimental (treatment) group for the given individual animal, and $\text{mean}(C_p^*(t)/Cp(t))_{\text{control group}}$ represents the mean plasma ratio of the chosen control group. Adapted from Dawson et al. (2008).

over a 10-sec period before being returned to their home cage. At exactly 45 min after isotope injection, animals were decapitated and a terminal blood sample collected by torso inversion. The brain was rapidly dissected out intact, frozen in isopentane (−40°C), and stored at −80°C until sectioning. Blood samples were centrifuged to separate the plasma, and aliquots were removed for the determination of plasma glucose (10 μl) and ^{14}C (20 μl) concentrations by semiautomated glucose oxidase assay (Beckman Glucose Analyzer) and liquid scintillation analysis (Packard, Tricarb 2900TL), respectively.

Frozen brains were sectioned (20 μm) in the coronal plane in a cryostat. A series of three consecutive sections were retained from every 120 μm, thaw mounted, and rapidly dried on a hot plate (70°C). Autoradiograms were generated by apposing these sections, together with precalibrated ^{14}C-standards (40–1,069 nCi/mg tissue equivalents; Amersham International, UK) to X-ray film (Kodak, SB-5) for 7 days. Autoradiographic images were analyzed by a computer-based image analysis system (MCID/M5+). The local isotope concentration for each brain RoI was derived from the optical density (OD) of autoradiographic images relative to that of the coexposed ^{14}C standard. Measurements were taken from 46 anatomically distinct brain regions defined with reference to a stereotactic mouse brain atlas (Franklin and Paxinos, 1997). iLCMRglu was calculated from the ^{14}C-isotope concentration in each brain region and the terminal plasma [^{14}C]/[glucose] ratio in accordance with the operational equation (Fig. 1) for this methodology. The use of this analytical approach has previously been shown to provide a valid measure of cerebral metabolism (Dawson et al., 2008), which parallels measurements gained by using the originally method outlined by Sokoloff et al. (1977). The experimental procedure applied here is also less invasive and reduces the stress experienced by the animals by avoiding the requirement for both intravascular cannulation and prolonged restraint, which are necessary in the original methodology.

Statistical Analysis

Data from [^{3}H]paroxetine binding and constitutive 2-deoxyglucose studies were analyzed by two-way analysis of variance (ANOVA) with sex (male, female) and genotype (wild type, *h*SERT OVR) as dependent variables. Within both studies, a significant genotype effect was analyzed within sex by two-way ANOVA with Bonferroni post hoc correction for multiple comparisons. In iLCMRglu studies, in keeping with previous studies of similar experimental design, anatomically discrete brain regions were assumed to represent variables that can be analyzed independently within each measure (McCulloch et al., 1982), and no correction was applied for multiple comparisons. In addition, the influence of sex on the ability of *h*SERT overexpression to alter the global level of cerebral metabolism was analyzed by the Mann-Whitney *U*-test comparison of the difference (ratio) between *h*SERT OVR and wild-type mice (within sex). Plasma variables for the 2-deoxyglucose study were analyzed by Student's *t*-test with Bonferroni post hoc correction applied for multiple comparisons. Significance was set at $P < 0.05$ throughout.

RESULTS

SERT Binding Sites in Male and Female *h*SERT OVR Mice

The distribution and concentration of [^{3}H]paroxetine binding in this study was similar to that reported by others in the brains of rats (DeSouza and Kuyatt, 1987; Reader et al., 1998; Sharkey et al., 1991). In all animals, [^{3}H]paroxetine binding was highest in the dorsal and median raphé, the molecular layer of the hippocampus, amygdaloid nuclei, hypothalamus, and the substantia nigra. Medium levels of binding were evident in subregions of the hippocampus and the nucleus accumbens, whereas relatively low levels of binding were detected in the striatum and anterior cortical regions [e.g., medial prefrontal cortex (mPFC), anterior cingulate, and frontal cortex].

In the majority of brain regions, [^{3}H]paroxetine binding tended to be greater in females than in males. Indeed, [^{3}H]paroxetine binding was found to be significantly higher in females compared with males in 19 of the 42 brain regions analyzed. This effect was particularly prevalent in cortical regions (including the dorsal mPFC +65%, posterior cingulate +97%, and somatosensory cortex +46%), the hippocampus (including the dentate PO +53%, CA2 +30%, ventral CA1 +51%), components of the basal ganglia (medial striatum +27%, globus pallidus +70%) and thalamic regions (mediodorsal +47%, ventrolateral +173%) (Fig. 2, Table I). In contrast, there was no evidence for a significant sex difference in [^{3}H]paroxetine binding in any of the amygdaloid or raphé nuclei.

In both male and female animals, the density of [^{3}H]paroxetine binding tended to be greater in *h*SERT OVR mice compared with wild-type animals in the majority of brain areas. In males, [^{3}H]paroxetine binding was significantly increased in *h*SERT OVR mice in 5 of the 42 brain regions analyzed (Fig. 2, Table I). In

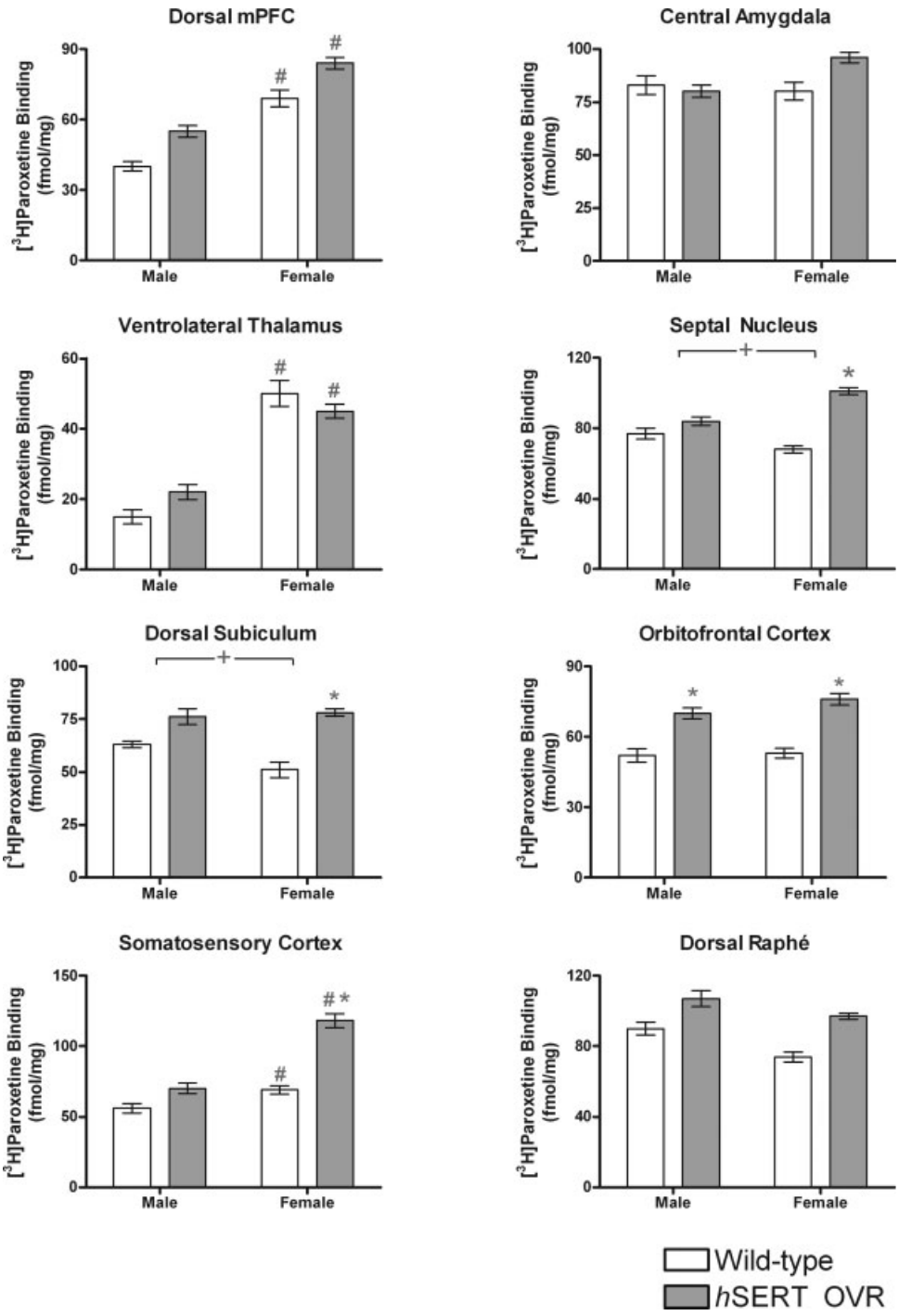

Fig. 2. [^{3}H]Paroxetine binding (fmol/mg tissue) in eight representative brain regions of wild-type and *h*SERT OVR male and female mice. Data were analyzed by two-way ANOVA. $^{\star}P < 0.05$ significant genotype effect (within sex with Bonferroni correction applied). $^{\#}P < 0.05$ significant sex effect. $^{+}P < 0.05$ significant sex × genotype interaction.

females, significant increases in [^{3}H]paroxetine binding in *h*SERT OVR mice were more widespread, occurring in 12 brain regions. This suggests that sex may modulate the ability of *h*SERT overexpression to increase SERT protein levels, with a greater effect being observed in females than in males. Indeed, a significant sex × genotype interaction was found in the septal nucleus ($F_{1,32} = 4.37$, $P < 0.05$) and dorsal subiculum ($F_{1,34} = 4.85$, $P < 0.05$), where the ability of *h*SERT overexpression to increase [^{3}H]paroxetine binding was significantly more marked in females than in males.

Interestingly, [^{3}H]paroxetine binding was not significantly altered in *h*SERT OVR animals in a number of brain regions, including the piriform cortex (+8%) or any of the amygdaloid nuclei (range −5% to +8%), indicating that increased SERT protein expression in *h*SERT OVR mice is regionally heterogenous (Fig. 2, Table I).

TABLE I. [^{3}H]Paroxetine Binding in *h*SERT OVR and Wild-type Mice[†]

	Male							Female						
	Wild type			*h*SERT OVR			%	Wild type			*h*SERT OVR			%
Site	Mean		SEM	Mean		SEM	Difference	Mean		SEM	Mean		SEM	Difference
Cortex														
Orbitofrontal	52	±	7	70	±	6★	35	53	±	5	76	±	6★	43
Frontal	72	±	9	87	±	6	21	66	±	6	82	±	6	23
Anterior cingulate	38	±	6	57	±	9	52	50	±	6#	81	±	7#,★	60
Dorsal medial prefrontal	40	±	5	55	±	6	37	69	±	9#	84	±	6#	22
Ventral medial prefrontal	45	±	5	68	±	5★	50	73	±	5#	83	±	8#	13
Somatosensory	56	±	8	70	±	9	24	69	±	7#	118	±	12#,★	71
Temporoparietal	30	±	8	59	±	7★	99	53	±	6	78	±	7	45
Posterior cingulate	18	±	5	34	±	5	94	31	±	6#	75	±	9#,★	140
Piriform	88	±	9	101	±	11	14	84	±	12	86	±	12	2
Entorhinal	87	±	9	88	±	10	2	72	±	4	96	±	8	33
Basal ganglia														
Medial striatum	47	±	6	63	±	6	34	55	±	6#	87	±	9#,★	59
Lateral striatum	34	±	7	47	±	9	37	36	±	5#	68	±	6#,★	85
Globus pallidus	35	±	6	58	±	7★	66	74	±	4#	86	±	9#	17
Subthalamic nucleus	42	±	6	45	±	9	7	37	±	5	32	±	5	−13
Substantia nigra pars reticulata	88	±	5	102	±	9	16	113	±	11#	120	±	12#	7
Substantia nigra pars compacta	74	±	6	91	±	7	22	90	±	4	113	±	12	25
Amygdala														
Medial	86	±	12	82	±	7	−5	91	±	13	103	±	9	13
Basolateral	87	±	12	83	±	7	−4	99	±	11	92	±	7	−7
Central	83	±	11	80	±	7	−4	80	±	10	96	±	6	20
Thalamic nuclei														
Anterior	56	±	5	61	±	6	11	59	±	8	76	±	7★	28
Mediodorsal	43	±	7	51	±	4	21	56	±	7#	84	±	9#,★	51
Ventrolateral	15	±	5	22	±	5	5	50	±	9#	45	±	5#	−10
Hypothalamic nuclei														
Anterior	81	±	8	88	±	5	8	93	±	8#	114	±	11#	21
Ventrolateral	69	±	7	84	±	5	21	84	±	9	101	±	16	19
Hippocampal														
Molecular layer	103	±	6	101	±	7	−2	103	±	13	105	±	7	2
Dorsal subiculum	63	±	4	76	±	9	20	56	±	9	78	±	4★,+	40
Dentate PO	22	±	7	36	±	6	58	38	±	3#	48	±	6#	27
Dorsal CA1	48	±	6	61	±	5	27	61	±	5#	80	±	4#,★	31
CA2	66	±	6	70	±	6	5	85	±	8#	92	±	8#	8
Ventral CA1	63	±	8	67	±	5	6	92	±	8#	104	±	8#	13
Ventral subiculum	79	±	7	89	±	7	13	90	±	9	96	±	8	6
CA3	56	±	6	64	±	7	14	63	±	6#	80	±	6#	27
Raphé														
Dorsal	90	±	9	107	±	11	19	74	±	7	97	±	4	32
Median	60	±	8	84	±	8	40	59	±	4	81	±	7	38
Paramedian	47	±	9	58	±	8	23	53	±	5	56	±	4	6
Mesocorticolimbic system														
Ventral tegmental area	30	±	5	53	±	7★	79	53	±	5#	69	±	9#,★	30
Nucleus accumbens	57	±	4	70	±	3	23	71	±	5	73	±	7	3
Multimodal														
Septal nucleus	77	±	8	84	±	6	8	68	±	5	101	±	5★,+	48
Bed nucleus of the stria terminalis	55	±	5	71	±	9	28	82	±	6#	94	±	11#	15
Lateral habenula	52	±	8	61	±	6	18	63	±	5	45	±	8	−28
Periaqueductal gray	68	±	8	78	±	7	13	76	±	12	93	±	8	22

[†]Effect of *h*SERT overexpression on [^{3}H]paroxetine binding (fmol/mg tissue) in the central nervous system. Data are expressed as mean ± SEM and percentage change in *h*SERT OVR mice compared with wild-type animals of the same sex. Data were analyzed by two-way ANOVA.
#$P < 0.05$ sex difference.
★$P < 0.05$, significant genotype effect (within sex with Bonferroni correction).
+$P < 0.05$ significant sex × genotype interaction.

Constitutive iLCMRglu

In all brain regions, iLCMRglu tended to be lower in females than in males, and this was confirmed by the significant sex effect observed in 33 of the 46 brain regions analyzed. The most marked difference between the sexes was present in the anterior hypothalamus (mean 40% lower in females). iLCMRglu was significantly lower in females compared with males in all hippocampal subregions (minimum effect −14%, maximum effect −26%) as well as in all amygdala (minimum −23%, maximum −26%) and raphé (minimum −21%, maximum −26%) nuclei.

In males, there was no evidence for a significant effect of *h*SERT OVR on iLCMRglu in any RoI. In contrast, in females, *h*SERT overexpression produced a significant reduction in iLCMRglu in 34 of the 47 brain regions analyzed. In females, significant decreases in iLCMRglu were found in all cortical regions (range, −31% in piriform cortex to −44% in frontal cortex), raphé nuclei (−36/37%), amygdala nuclei (range, −30% in central to −36% in basolateral), and in multiple hippocampal subfields (range, −34% in dorsal subiculum to −38% in the dorsal CA1) (Table II). This suggested that the ability of *h*SERT OVR to modify constitutive LCMRglu was influenced by sex. Indeed, the effect of *h*SERT OVR on the global level of cerebral metabolism was significantly greater in females than in males (Mann-Whitney *U*-test). Furthermore, when looking at each brain RoI independently a significant sex × genotype interaction was detected in 7 of the 46 brain regions analyzed (Fig. 3). These were detected in the medial striatum (26% greater reduction in females; $F_{1,39} = 4.34$, $P < 0.05$), globus pallidus (25% greater reduction in females; $F_{1,38} = 4.39$, $P < 0.05$), ventrolateral thalamus (32% greater reduction in females; $F_{1,39} = 5.52$, $P < 0.05$), and the mammillary body (27% greater reduction in females; $F_{1,32} = 5.86$, $P < 0.05$).

The terminal plasma ^{14}C and glucose concentrations for *h*SERT OVR and wild-type mice involved in the iLCMRglu study are shown in Table III. There was no evidence for a significant difference in these plasma variables between *h*SERT OVR and wild-type mice in either sex. Significant sex differences in terminal plasma glucose and ^{14}C levels, the former of which are at the lower end of the normal physiological range as a result of the overnight food restriction implemented in these studies, most likely results from the lower body weight of females compared with males. These differences result in a significant and appropriate sex-based adjustment of the plasma [^{14}C]:[glucose] ratio for use in the determination of iLCMRglu and so differences in these plasma variables are unlikely to contribute to the sex differences in iLCMRglu found in this study.

DISCUSSION

Given the known influence of sex in affective disorders and reported sex differences in serotonergic system function (for review, see Rubinow et al., 1998), there is a paucity of data on sex differences in SERT expression. However, there is evidence that the density of high-affinity [^{3}H]imipramine binding sites is greater in the brain of female rats (Ieni et al., 1985) and that β-CIT (2-beta-carboxymethoxy-3beta-(4-iodiphenyl)tropane), which labels both SERT and the dopamine transporter (DAT), is higher in human females than males (Staley et al., 2001). This provides some indirect support for our data showing significantly higher [^{3}H]paroxetine binding to SERT in a number of diverse brain regions in female mice when compared with males. Although the sex steroids may have a role in determining observed sex differences in SERT protein expression, the exact relationship may not be simple. Thus, although exogenous estrogen has been found to stimulate both SERT gene transcription and protein expression levels (McQueen et al., 1997), the effects are highly localized in the brain, and the pattern does not match the regional distribution of sex differences found in naturally cycling mice in our study. Furthermore, testosterone in males has been found to stimulate SERT expression (McQueen et al., 1999) almost as much as estrogen does in females. Therefore, it seems unlikely that sex differences in SERT expression are determined solely by the acute regulatory effects of the sex steroids. Whatever the mechanism, sex differences in SERT protein expression and function are likely to contribute to some of the sexual dimorphisms reported in functions associated with the 5-HT system, including sex differences in affective function and antidepressant efficacy. In particular, the enhanced expression of SERT in females may prove to be central to the decreased levels of synaptic 5-HT found in females compared with males (Gundlah et al., 1998; Jones and Lucki, 2005; Mitsushima et al., 2006).

Applying the same radioligand imaging that we used in wild-type mice, we found that although [^{3}H]paroxetine binding was increased in *h*SERT OVR mice, the regional distribution of binding was qualitatively similar to that found in wild-type mice, as reported previously (Jennings et al., 2006). Furthermore, the pattern was essentially similar to that described using a variety of selective SERT radioligands in rats (DeSouza and Kuyatt, 1987; Reader et al., 1998; Sharkey et al., 1991). The expression of both murine and human SERT proteins in *h*SERT OVR mice results in the presence of more than one pharmacologically defined binding site for SERT ligands (Loder et al., 2000), presumably as a result of the differing affinities of murine compared with the human transporter for these ligands (Plenge and Mellerup, 1991). However, the use of a saturating concentration of radioligand in our studies for both species of SERT proteins allows us to suggest that it is increased SERT protein expression (B_{max}) rather than altered affinity that accounts for the increased [^{3}H]paroxetine binding found in *h*SERT OVR mice. Interestingly, we also found that the reported enhancement of SERT gene transcription, as reflected by the increase in SERT mRNA observed in the raphé nuclei of *h*SERT OVR mice (Jennings et al., 2006), does not result in a universal increase in SERT protein expression

TABLE II. Constitutive iLCMRglu in *h*SERT OVR and Wild-type Mice[†]

	Male			Female		
Site	Wild type	*h*SERT OVR	%	Wild type	*H*sert OVR	%
Cortex						
Orbitofrontal	58 ± 6	50 ± 5	−14	43 ± 4$^{\#}$	31 ± 3$^{\#,\star}$	−36
Frontal	43 ± 5	36 ± 4	−16	44 ± 3	25 ± 2*	−44
Anterior cingulate	46 ± 5	38 ± 4	−15	43 ± 3	27 ± 3*	−38
Dorsal medial prefrontal	44 ± 6	35 ± 3	−21	37 ± 3$^{\#}$	23 ± 2$^{\#,\star}$	−39
Ventral medial prefrontal	40 ± 4	31 ± 3	−21	29 ± 2$^{\#}$	17 ± 2$^{\#,\star}$	−40
Somatosensory	51 ± 5	46 ± 5	−9	55 ± 5	28 ± 3*	−44
Temporoparietal	51 ± 5	47 ± 5	−8	44 ± 4$^{\#}$	28 ± 2$^{\#,\star}$	−35
Posterior cingulate	49 ± 5	47 ± 5	−6	48 ± 4	32 ± 3*	−34
Piriform	29 ± 3	24 ± 2	−17	20 ± 2$^{\#}$	14 ± 2$^{\#,\star}$	−31
Entorhinal	34 ± 3	30 ± 3	−14	30 ± 2$^{\#}$	19 ± 2$^{\#}$	−35
Basal ganglia						
Medial striatum	41 ± 3	38 ± 4	−7	42 ± 3	25 ± 3$^{\star,+}$	−40
Lateral striatum	46 ± 4	40 ± 4	−12	42 ± 3$^{\#}$	26 ± 3$^{\#,\star}$	−37
Globus pallidus	30 ± 2	28 ± 3	−9	33 ± 3	18 ± 2$^{\star,+}$	−44
Subthalamic nucleus	46 ± 5	45 ± 5	−2	50 ± 4	30 ± 3*	−34
Substantia nigra pars reticulata	29 ± 2	27 ± 2	−7	26 ± 2$^{\#}$	17 ± 2$^{\#,\star}$	−35
Substantia nigra pars compacta	36 ± 3	33 ± 3	−9	34 ± 3$^{\#}$	21 ± 2$^{\#,\star}$	−39
Amygdala						
Medial	24 ± 2	21 ± 2	−14	18 ± 2$^{\#}$	12 ± 2$^{\#}$	−32
Basolateral	33 ± 2	30 ± 3	−9	29 ± 2$^{\#}$	18 ± 2$^{\#,\star}$	−36
Central	22 ± 2	19 ± 2	−12	17 ± 2$^{\#}$	12 ± 2$^{\#}$	−30
Thalamic nuclei						
Anterior	47 ± 4	47 ± 5	0	47 ± 5	32 ± 3	−32
Mediodorsal	54 ± 5	47 ± 4	−14	48 ± 4$^{\#}$	29 ± 3$^{\#,\star}$	−39
Ventrolateral	53 ± 5	50 ± 5	−6	56 ± 5	31 ± 3$^{\star,+}$	−46
Hypothalamic nuclei						
Anterior	37 ± 6	34 ± 4	−9	19 ± 3$^{\#}$	13 ± 2$^{\#}$	−34
Ventrolateral	28 ± 2	27 ± 3	−3	26 ± 2$^{\#}$	16 ± 2$^{\#,\star}$	−37
Hippocampus						
Molecular layer	41 ± 4	34 ± 3	−16	32 ± 3$^{\#}$	21 ± 2$^{\#,\star}$	−35
Dorsal subiculum	38 ± 3	34 ± 3	−11	38 ± 3	25 ± 3*	−34
Dentate PO	25 ± 1	22 ± 2	−11	20 ± 2$^{\#}$	15 ± 2$^{\#}$	−25
Dorsal CA1	35 ± 3	30 ± 2	−16	28 ± 3$^{\#}$	17 ± 2$^{\#,\star}$	−38
CA2	33 ± 3	27 ± 3	−18	26 ± 3$^{\#}$	16 ± 2$^{\#,\star}$	−38
Ventral CA1	33 ± 2	28 ± 3	−16	24 ± 2$^{\#}$	17 ± 2$^{\#}$	−31
Ventral subiculum	29 ± 2	26 ± 3	−11	23 ± 2$^{\#}$	17 ± 2$^{\#}$	−26
CA3	27 ± 2	24 ± 2	−14	21 ± 2$^{\#}$	14 ± 2$^{\#}$	−34
Raphé						
Dorsal	36 ± 3	31 ± 3	−14	29 ± 2$^{\#}$	18 ± 2$^{\#,\star}$	−37
Median	46 ± 5	39 ± 4	−15	39 ± 3$^{\#}$	25 ± 3$^{\#,\star}$	−36
Paramedian	45 ± 4	39 ± 4	−13	40 ± 3$^{\#}$	25 ± 3$^{\#,\star}$	−37
Mesocorticolimbic system						
Ventral tegmental area	36 ± 3	37 ± 5	2	43 ± 4	28 ± 3*	−35
Nucleus accumbens	43 ± 6	34 ± 3	−21	31 ± 2$^{\#}$	19 ± 2$^{\#,\star}$	−40
Multimodal						
Septal nucleus	34 ± 3	29 ± 3	−13	29 ± 2$^{\#}$	18 ± 2$^{\#,\star}$	−37
BNST	27 ± 3	21 ± 2	−21	17 ± 2$^{\#}$	11 ± 2$^{\#}$	−35
Lateral habenula	53 ± 5	48 ± 5	−10	55 ± 4	32 ± 3*	−41
Mamillary body	52 ± 4	50 ± 5	−3	56 ± 3	34 ± 3$^{\star,+}$	−39
Periaqueductal grey	33 ± 3	26 ± 2	−20	23 ± 3$^{\#}$	15 ± 2$^{\#}$	−34
Inferior colliculus	73 ± 7	71 ± 10	−3	81 ± 7	48 ± 5	−40†
Ventral tegmental nucleus	46 ± 4	41 ± 4	−12	43 ± 3$^{\#}$	26 ± 2$^{\#,\star}$	−40
Locus coeruleus	41 ± 4	36 ± 4	−13	38 ± 3$^{\#}$	22 ± 2$^{\#,\star}$	−42
Nucleus tractus solitaris	56 ± 9	44 ± 7	−20	37 ± 4$^{\#}$	17 ± 1$^{\#}$	−52

[†]Constitutive iLCMRglu (nCi·mg^{-1}/nCi·μmol^{-1}) in male and female *h*SERT OVR and wild-type mice. Data shown as mean ± SEM iLCMRglu and % change in *h*SERT OVR mice compared with wild-type animals of the same sex. Data were analyzed by two-way ANOVA.

$^{\#}P < 0.05$ significant sex effect.

$^{\star}P < 0.05$ significant *h*SERT effect within sex (two-way ANOVA with Bonferroni correction).

$^{+}P < 0.05$ significant sex × genotype interaction.

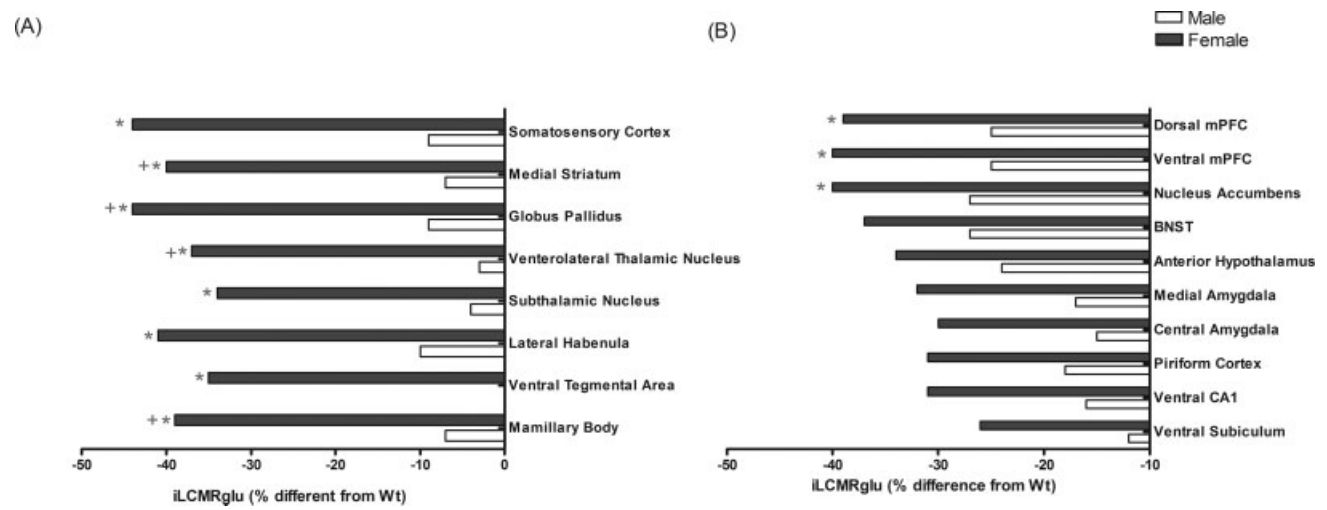

Fig. 3. Effect of *h*SERT OVR on constitutive iLCMRglu in male and female mice. Data shown as percentage difference in the iLCMRglu from appropriate wild-type control. Representative regions are shown for the (**A**) maximum and (**B**) minimum sex difference in the effect of *h*SERT overexpression on iLCMRglu. ⋆$P < 0.05$ significant *h*SERT effect (within-sex Bonferroni correction). $^{+}P < 0.05$ significant sex × *h*SERT OVR interaction (two-way ANOVA).

TABLE III. Plasma Glucose and ^{14}C in *h*SERT OVR and Wild-type Mice[†]

	Male		Female	
Variable	Wild type	*h*SERT OVR	Wild type	*h*SERT OVR
Plasma glucose ($\mu mol \cdot ml^{-1}$)	9.03 ± 0.61	7.28 ± 0.80	6.52 ± 0.44⋆	7.68 ± 0.49
Plasma [^{14}C] ($nCi \cdot ml^{-1}$)	77.47 ± 4.53	75.69 ± 8.55	96.05 ± 13.57⋆	138.48 ± 18.12⋆
Plasma [^{14}C]:[glucose] ($nCi \cdot \mu mol^{-1}$)	8.41 ± 0.65	11.89 ± 1.95	15.34 ± 2.11⋆	18.02 ± 2.41⋆

[†]Terminal plasma ^{14}C ($nCi \cdot ml^{-1}$) and glucose ($\mu mol \cdot ml^{-1}$) concentrations in wild-type and *h*SERT OVR mice. Data are mean ± SEM.
⋆$P < 0.05$ significant sex difference (within genotype; Student's *t*-test with Bonferroni correction).

throughout the brain. Rather, SERT expression is enhanced in a regionally heterogenous manner, with the greatest increase found in anterior cortical regions, moderate increases in components of the basal ganglia, hippocampal regions, and raphé, while in other regions, such as the amygdala nuclei, no increase is observed. This suggests that the increased SERT protein expression in *h*SERT OVR mice is not directly related to the enhancement of SERT gene transcription. Rather, complicated regulatory mechanisms must exist that govern SERT protein expression levels, and these must operate in a brain region–specific manner. As yet, the nature of these mechanisms is not understood, but because they will directly regulate the outcome of enhanced SERT gene transcription, they are likely to be central in mediating altered serotonergic function in *h*SERT OVR mice. The same mechanisms may also mediate the effects of the 5-HTTLPR polymorphism on brain and affective functioning in humans.

The approximate 2-fold increase in SERT binding, in specific brain regions of *h*SERT OVR mice, shows a striking parallel to the magnitude of increase (also approximately 2-fold) in SERT binding, mRNA expression, and 5-HT reuptake found in both brain and blood platelets of human individuals with the l/l genotype over those of the s/l or s/s genotype (Greenberg et al., 1999; Heinz et al., 2000; Little et al., 1998). This adds further credence to the suggestion that *h*SERT OVR mice provide a useful model of the natural variation in SERT protein expression observed in humans—for example, as a result of the 5-HTTLPR polymorphism. However, it is also important to note that a number of other studies have reported that there is no effect of the 5-HTTLPR polymorphism on SERT binding in the human brain (Parsey et al., 2006; Shioe et al., 2003; Van Dyck et al., 2004; Willeit et al., 2001). Although it is interesting to speculate, with reference to our results, that the regional localization of the binding measurements made in some of these studies may be responsible for their negative findings, it is also likely that the use of small sample sizes in some of these studies might contribute to their negative results. More importantly, because our results suggest that the ability of a genetically determined increase in SERT transcription increases SERT protein expression is strongly influenced by sex, with greater increases occurring in females than in males, lack of sample segre-

gation on the basis of sex in human studies investigating the 5-HTTLPR polymorphism may be a major confounding factor resulting in their negative results.

In this study, we found that a lifelong increase in SERT function decreases constitutive cerebral metabolism in a number of brain regions, and that this effect was only significant in females. Interestingly, many of the regions in which a decreased rate of iLCMRglu was found in *h*SERT OVR females compared with wild-type animals are strongly implicated in the regulation of anxiety. Pharmacological challenges associated with an anxiolytic effect, including acute diazepam and phenobarbital treatment, commonly result in decreased LCMRglu in many of the regions in which constitutive iLCMRglu is decreased in *h*SERT OVR mice. These regions include the mamillary body, ventrolateral thalamus, septal nucleus, medial striatum, and globus pallidus (Ableitner et al., 1985; Ableitner and Herz, 1987; Kelly et al., 1986). In addition, a number of anxiogenic pharmacological challenges have been reported to elicit increased LCMRglu in these structures (Pratt et al., 1988; Ableitner and Herz, 1987) and chronic prenatal treatment with diazepam, which produces an anxiolytic-like phenotype in the ''drug-free'' adult, is also associated with reduced LCMRglu in many of these brain regions (Schroeder et al., 1997).

Our data suggest that constitutive cerebral function is decreased in several brain structures in which neuronal activity is positively correlated with anxiety. In addition, elevated LCMRglu in the lateral habenula appears to be the most robust correlate of depressive behavior in rats, and this hypermetabolism is reversed, in parallel with the alleviation of depressive-like behavior, by antidepressant treatment (Caldecott-Hazard et al., 1988). In this context, it is worth noting that iLCMRglu is also significantly decreased in the lateral habenula of *h*SERT OVR mice, suggesting that hypometabolism in this region may contribute to their depression-resistant phenotype. In a wider context, the observation of decreased constitutive metabolism in limbic structures of female *h*SERT OVR mice parallels the reported basal frontolimbic hypometabolism reported in l/l compared with s/s allele human individuals (Graff-Guerrero et al., 2005). However, in contrast to the observation that hypometabolism is limited to frontal cortical structures in l/l human individuals, we found evidence for a more widespread effect in *h*SERT OVR female mice, which also included many subcortical regions and the raphé nuclei. One reason for this disparity may be the lack of sample segregation on the basis of sex in the human study, which could lead to the masking of a more widespread hypometabolism in l/l allele females by the presence of a smaller effect in males.

Evidence from both [^{3}H]paroxetine binding and constitutive iLCMRglu data suggests that sex modulates the influence of a lifelong increase in SERT transcription on these parameters, with a greater effect in females than in males. The greater increase in [^{3}H]paroxetine binding induced by *h*SERT overexpression in females compared with males suggests that direct sex modulation of the increase in SERT protein expression elicited in response to increased SERT gene transcription may be central to the sex-dependent effects of the 5-HTTLPR polymorphism on affective functioning. However, in *h*SERT OVR mice the modulatory influence of sex on increased SERT expression was limited in its regional distribution, being significant in the dorsal subiculum and septal nucleus. In contrast, the influence of sex on the cerebral hypometabolism present in *h*SERT OVR animals was more widespread and did not include either the dorsal subiculum or septum. Furthermore, the regions in which iLCMRglu was altered by *h*SERT OVR to a greater extent in females than in males do not receive dense innervation from either the dorsal subiculum or septum. Thus, in terms of anatomical distribution, the effect of sex on the ability of *h*SERT overexpression to induce cerebral hypometabolism does not directly map onto the effect of sex on the altered SERT expression levels in these animals. However, it is commonly reported that regional alterations in LCMRglu in response to pharmacological challenge do not correlate with the regional distribution of receptors. This is thought to be due to both the sensitivity of the method, which localizes metabolic alterations at the synapse rather than cell body and to the convergence and contribution of polysynaptic pathways to the observed alterations in LCMRglu. Therefore, it is not surprising that the sex effects of *h*SERT OVR on SERT protein expression show little spatial correlation with those of iLCMRglu. Furthermore, sex-dependent modulation of any alteration in 5-HT receptor functioning that may occur in response to the altered SERT function present in *h*SERT OVR animals may also contribute to the differences noted in iLCMRglu. Such a mechanism has been found to exist in SERT KO mice where alteration in 5-HT_{1A} receptor function are found in female, but not male, animals (Li et al., 2000).

Overall, these data suggest that a lifelong increase in SERT transcription modifies constitutive cerebral function to a greater extent in females than in males. Although this finding parallels reported sex differences observed in humans in the effect of the 5-HTTLPR polymorphism on affective functioning, the possible sex difference in anxiety-like behavior in *h*SERT OVR animals has not been investigated. However, our data suggest that *h*SERT overexpression may produce a greater anxiolytic effect in females than in males. In summary, alterations in SERT expression and cerebral function in *h*SERT OVR mice parallel those observed in l/l allele human individuals, suggesting that *h*SERT OVR mice provide a useful animal model of the 5-HTTLPR polymorphism. In addition, these alterations are greater in females than in males, which parallels the known sex difference in the effect of the 5-HTTLPR polymorphism on affective functioning in humans.

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